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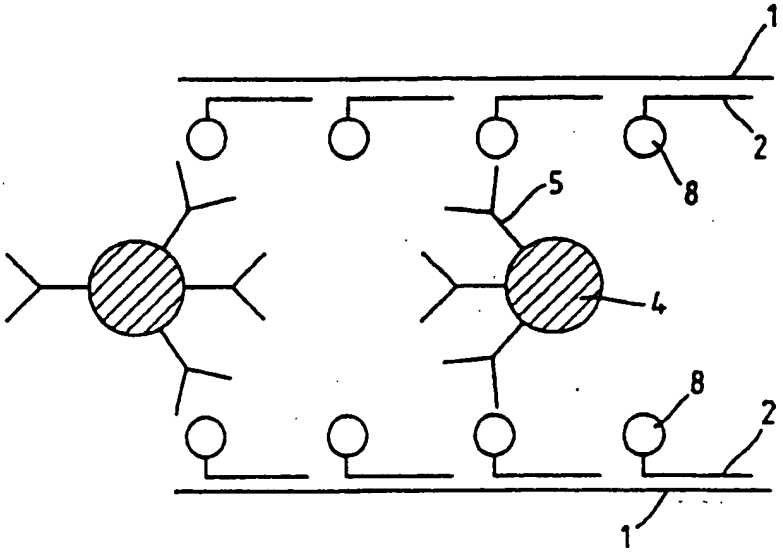


(57) Abstract

A method to detect the presence of nucleic acid sequences by agglutination of a generic particle reagent. Known methods require a specific particle reagent for each sequence to be detected. In the presence of a given nucleic acid sequence a nucleic acid species is formed which comprises at least two binding partners. The generic particle reagent is coated with a specific binding partner which can bind to the binding partners in the nucleic acid species and thus lead to agglutination of the particle. The method may also be used to detect mutati ns in a nucleic acid sequence. A preferred embodiment uses a hapten such as fluorescein as the binding partner in the nucleic acid species and anti-hapten antibody coated particles such as anti-fluorescein antibody coated latex as the generic particle reagent.

NUCLEIC ACID DETECTION METHOD USING PARTICLE AGGLUTINATION

The present invention relates to the detection of specific target nucleic acid sequences using particle agglutination, and in particular to the use of generic particle reagents to detect the presence or absence of many different target nucleic acid sequences.

Many of the methods presently available for detecting nucleic acid sequences are relatively complex procedures and are laborious to perform. Examples are described in Hybridisation, by B.D. Hames and S.J. Higgins (Eds) IRL Press 1985 and J.A. Matthews et al, Analytical Biochemistry, 169, 1988, 1-25 (Academic Press). A typical method involves binding the nucleic acid sequence to be assayed to a solid support such as nylon. This is probed with a complementary nucleic acid sequence containing a label. Any probe not bound to the target is washed away. Any label remaining bound to the solid support is detected by appropriate means. For example if the label was ^{32}P it could be detected by autoradiography or if it was an enzyme then an enzyme substrate could be applied to develop a signal such as colour.

Alternative methods involving the use of capture probes are also known. Capture probes are labelled with a specific binding partner which is used to immobilise the specific nucleic acid sequence on a solid phase such as a microtiter well. These capture probes are used in conjunction with signal probes containing a detectable label such as ^{32}P or an enzyme as described above. Only when both the capture and signal probes bind to the specific sequence to be assayed does signal remain bound to the solid phase after washing to remove unbound probes.

K. Kleppe et al in J. Mol. Biol. (1971). 56, 341-361 disclose a method for the amplification of a desired DNA sequence. The method involves denaturation of a DNA duplex to form single strands. The denaturation step is carried out in the presence of a sufficiently large excess of two nucleic acid primers which upon

cooling hybridise to regions defining the desired DNA sequence. Two structures are thus obtained each containing the full length of the template strand appropriately complexed with primer. DNA polymerase and a sufficient amount of each required deoxynucleoside triphosphate are added whereby two molecules of the original duplex are obtained. The above cycle of denaturation, primer annealing, and extension are repeated until the appropriate number of copies of the desired DNA sequence is obtained. The above method is now referred to as the polymerase chain reaction (PCR), as described for example in EP 0 201 184. In addition to the methods outlined above for detecting nucleic acid sequences, DNA amplified by PCR can be detected using electrophoresis. The presence of a band of the expected size as defined by the primers is taken as evidence of the specific sequence in the material assayed. This method is also relatively time consuming and laborious.

It is desirable to detect specific nucleic acid sequences in the most convenient manner. A convenient detection method involves agglutination of particles, preferably latex particles. This has been applied extensively to the detection of proteins and haptens as described in Uniform Latex Particles by L.B. Bangs, published in October 1984, Seradyn Inc., PO Box 1210, Indianapolis, IN 46206, USA. Pages 51-58 are the most relevant. For example, in order to detect a macromolecule such as a protein, latex particles are coated with specific antibodies against the protein. In the presence of the specific protein the latex particles will agglutinate due to the protein molecules acting as a link between the latex particles.

A method of detecting nucleic acid sequences using particle agglutination has been described in PCT WO 87/05334. This method depends upon having assay specific nucleic acid sequences bound to the particles. In the presence of target nucleic acid sequences complementary to those on the particles agglutination will occur. Alternatively the particles themselves can be coated with nucleic acid sequences complementary to each other so that agglutination will occur spontaneously. If complementary sequences are present in a sample to

be assayed then the spontaneous agglutination of the particles will be inhibited. A disadvantage of the above method is that specific nucleic acids must be bound to the particles for each nucleic acid sequence to be assayed. This is time consuming and laborious for the manufacturer. Furthermore the number of solid phase components is generally best kept to a minimum if a panel of tests using particulate solid phases is to be automated by for example instrumentation for liquid handling. Solid phase components often need to be mixed before use, making instrumentation more complex than simply dispensing liquids. The method disclosed in PCT WO/87/05334 requires a different particulate solid phase for each test which is a disadvantage for such automation. It is desirable to provide further methods for the detection of nucleic acid sequences utilising particle agglutination which overcome, at least in part, the disadvantages of the prior art.

The present invention is based on the discovery that a specific target nucleic acid sequence may be detected by particle agglutination utilising specific binding partner coated particles which do not directly hybridise to the target nucleic acid sequence. Thereby a generic particle reagent may be utilised to eliminate the need for the manufacturer to produce a specific solid phase for each nucleic acid sequence to be assayed. A further advantage of the invention is to simplify the process of automation of the assay method due to the reduction of the number of solid phase components needed for a panel of tests. The present invention has useful sensitivity to exploit the invention on convenient systems of detection such as spectrophotometers. Customer convenience may also be improved.

According to one feature of the present invention we provide a method for detecting the presence or absence of a target nucleic acid sequence in a sample by particle agglutination which method comprises contacting specific binding partner coated particles which do not directly hybridise to the target nucleic acid sequence with a species comprising nucleic acid which species is formed as a function of the presence or absence of the target nucleic acid and which also comprises at least two monovalent binding partners which may be the

same or different and detecting the presence or absence of particle agglutination mediated by said species. Preferably at least two target nucleic acid sequences, more preferably at least 3 target nucleic acid sequences, more preferably at least 4 target nucleic acid sequences and especially at least 5 target nucleic acid sequences are detected using only one specific binding partner coated particle.

The following glossary of terms used in the present specification is provided to assist the reader:-

The term "specific binding partner" as used herein relates to a member of any specific binding pair for example antigen/antibody, hapten/antibody, biotin/avidin, biotin/streptavidin, nucleic acid sequence/complementary nucleic acid sequence, sugar/lectin or compound/binding moiety (eg thyroxine/thyroxine binding globulin).

The term "antibody" as used herein includes whole antibodies and antibody fragments comprising at least part of the binding site for example Fab and Fv fragments (Methods in Enzymology 178, Academic Press 1989) and Dabs (Ward, E.S. et al., 1989 Nature 341, 544).

The term "generic particle" as used herein relates to the ability of the same particle reagent to be used for the assay of more than one specific nucleic acid sequence.

The term "monovalent binding partner" as used herein is defined as a member of a specific binding pair incapable of forming crosslinks between the particles coated with the other member of the specific binding pair, such that spontaneous agglutination of the particles in the absence of target nucleic acid sequence does not occur. The monovalent binding partner (mbp)is selected such that it cannot form a complex with more than one molecule of the other member of the specific binding pair. If it did it may be possible to form crosslinks between particles and agglutination would be possible in the absence of the specific nucleic acid sequence to be detected. Particularly convenient examples of monovalent binding partners (mbp) include fluorescein and biotin. If the monovalent binding partner is

of a size where it is sterically possible to bind more than one molecule of the corresponding binding partner this puts restrictions on the properties of the latter such that it can only bind to one site. For example if the monovalent binding partner is human chorionic gonadotrophin (hCG) then a suitable corresponding binding partner is a monoclonal antibody to a single epitope on hCG but not a polyclonal antibody recognising multiple epitopes. However in the case of a hapten such as fluorescein, polyclonal antibodies are adequate since only one molecule can bind for steric reasons. If the monovalent binding partner consists of a nucleic acid sequence this is preferably selected such that under the assay conditions it does not bind to sequences in the samples but only hybridises to the complementary sequence on the particles in a 1:1 ratio. For certain specific binding partner pairs the juxtaposition between particle and probe is important. For example, polyclonal anti-fluorescein antibody on particles and fluorescein on the probes is satisfactory but the converse would lead to spontaneous agglutination of particles in the absence of target.

The term "hybridised" as used herein includes hybridisation between a polynucleotide probe and a desired target sequence but excludes hybridisation between a polynucleotide probe and non-desired nucleotide sequences.

The term "elongation of a nucleic acid sequence" is defined as the formation of a nucleic acid polymer wherein at least one of the monomers incorporated into the newly synthesised polymer comprises a monovalent binding partner. Preferred examples include polymerase mediated extension by for example DNA or RNA polymerase enzymes with at least one nucleoside triphosphate labelled with a lov molecular weight mbp such as fluorescein, biotin or digoxigenin used as a substrate.

The term "specific binding partner coated particles" may comprise one or more types of particles coated with one or more types of specific binding partner. Preferably one type of particle will be

coated with one type of specific binding partner. Conveniently the particles may be coated with antibodies. Particularly preferred are polystyrene latex particles coated with immunoglobulin class G antibodies specific for a particular antigen or hapten.

There are a number of ways to detect particle agglutination ranging from simple visual examination through to sophisticated particle counting instrumentation commercially sold as PACIA (Collet-Cassart et al, Clinical Chemistry, 27, 64, 1981). This and other methods are discussed in the Bangs (1984) reference. The method of detection will depend upon the sensitivity required. If amplification techniques such as PCR are used to provide relatively high concentrations of target nucleic acid then expensive systems such as PACIA should not be required. In these cases it is highly desirable that agglutination be measured on instruments widely installed in laboratories at present. These include so-called clinical chemistry machines which have detectors for absorbance and sometimes nephelometry.

The method of the present invention is applicable to molecular biology research and to all areas of diagnostic medicine and other diagnostic sciences, for example forensic, agricultural, veterinary or food sciences where it is necessary to detect or measure specific nucleic acid sequences. In particular it is applicable to the detection of infectious micro-organisms and to the detection of mutations, deletions and rearrangements which give rise to various inherited diseases and predispositions. A further application of the invention is that it may be used to measure nucleic acids that are themselves generated as part of an assay method, for example the RNA sequence that is generated by Q-beta replicase as described in Nucleic Acids Research, 14, (1986), 5591-5603. A particularly advantageous use is for the assay of DNA sequences generated by PCR including the amplification refractory mutation system as described in our copending European patent application number 332435. Further advantageous uses are in relation to the transcription based amplification system of Siska Diagnostics as described in PCT WO 88/10315 and the ligase

mediated amplification system of Biotechnica International described in EP 320308. Alternatively it may be used to detect sequences occuring naturally in high copy numbers such as ribosomal RNA. Thus the present invention may be advantageously applied to a wide range of nucleic acid assay designs.

The prozone phenomenon is well understood by the ordinary scientist working in the field of agglutination assays and is discussed by Bangs (1984). This phenomenon principally relates to the presence of an excess of target over the reagents involved in the agglutination reaction. In these conditions the amount of target can be so great as to reduce the amount of agglutination compared with lower concentrations of target. The behaviour of such samples in an assay is that the degree of agglutination will increase upon dilution of the sample over a given range. Thereafter a plateau will be reached before the degree of agglutination becomes directly proportional to the target concentration. These samples can give rise to false negative results and therefore the assay components are preferably optimised to minimise this effect.

The particles can be any particulate insoluble material capable of being coated with a member of a specific binding pair. Examples include latex (see Bangs 1984 for a discussion of types of latex particles), charcoal, colloidal gold, glass, red blood cells and liposomes. Polystyrene latex particles are particularly convenient. The optimum properties of the particle will depend on the specific application. Generally the particles will be selected such that settling out during the assay is minimised for example by selecting the particles to have approximately the same density as the assay medium and the size of the particles will be chosen such that there is good discrimination between the agglutinated and non-agglutinated particles in the detection system. For example in an instrumented optical detection system the wavelength of light will have an effect on the optimal particle size and in a system involving filtration the unagglutinated particles will be selected to migrate through the filter matrix whereas the agglutinated particles will be trapped.

Preferably the particles will be in a size range of 0.01-10 µm, more preferably 0.1-5µm, conveniently 0.5-2.5µm, advantageously 0.7-0.9µm and especially 0.8µm.

The coating of the particles with specific binding partner may be by covalent or non-covalent means. As an example of non-covalent coating, antibodies are adsorbed onto the surface of polystyrene latex particles by mixing the components in an appropriate buffer and washing to remove unbound antibody. As an example of covalent coating, antibodies can be covalently linked to latex particles with carboxylic acid groups on their surface. Crosslinking agents such as water soluble carbodiimide can link the carboxylic acid groups on the latex to amino groups on antibodies. These and other approaches are discussed in the Bangs reference. Methods to coat nucleic acids onto particles are discussed in PCT WO 87/05334. These include direct covalent bonding to the particles or covalent bonding to molecules such as protein on the particles. In some cases it can be advantageous to incorporate components into the particles which aid the detection of agglutination. For example dyes can be incorporated into latex particles (Bangs, 1984). This may be advantageous where visual detection is employed for example using blue particles in a white filtration device. In some cases there will be some agglutinated particles present after the coating process or the particles may tend to agglutinate spontaneously on storage due to a natural affinity between the particles. These issues have been discussed in a Newsletter from Bangs Laboratories Inc, 979 Keystone Way, Carmel, IN 46032, USA in December 1989.

The sample is generally of biological origin, for example obtained from humans, such as blood, tissue, sputum and urine; or swabs taken from various sites on the body such as urogenital or throat.

Nucleic acid comprises DNA or RNA and the polynucleotide probes include polynucleotides of DNA, RNA or any other kind hybridisable to nucleic acid sequences. It will be appreciated that

such nucleic acid sequences may include base analogues as well as naturally occuring bases cytosine, adenine, guanine, thymine and uracil. Such base analogues include hypoxanthine, 2,6-diaminopurine and 8-azaguanine. The probes may be in double stranded (ds) or single stranded (ss) form but are preferably in single stranded form. Double stranded probes are denatured before use. They may be prepared by direct synthesis, polymerase mediated extension reactions or by cloning or any other convenient method.

According to a further feature of the present invention we provide a method for detecting the presence or absence of a target nucleic acid sequence as hereinbefore described wherein at least one of the monovalent binding partners has been incorporated into the species by hybridisation of a polynucleotide probe comprising one copy of a monovalent binding partner. A preferred embodiment of the invention comprises the hybridisation of at least two, and preferably more, of the polynucleotide probes to the target sequence. The polynucleotide probes are preferably oligonucleotides all labelled with the same monovalent binding partner (mbp) such as fluorescein. One type of particles are preferably used such as latex particles coated with anti-fluorescein antibodies. Agglutination of the particles can be detected by appropriate means. The advantage for the manufacturer of this embodiment is one of simplicity and cost-efficiency since only one particle reagent need be produced and oligonucleotide probes may be conveniently synthesised in adequate amounts on commercially available instruments.

Suitable conditions for hybridisation are well known to the scientist of ordinary skill. Convenient temperatures for hybridisation include ambient temperature, typically 20 to 25 degrees Centigrade, as well as other temperatures below the melting temperature of the probe and its complementary sequence. The pH of the reaction buffer is generally neutral or moderately alkaline, for example pH 7.5 to 9.0 and the ionic strength is chosen such that the desired hybridisation occurs but unwanted hybridisation with other sequences is excluded.

The distinguishing power of the probe is generally maximised by 100 per cent homology with the target but this is not essential provided that only the desired hybridisation can occur under the conditions employed. The conditions employed should not substantially interfere with the interaction between the specific binding partners on the particles and the probes. Probes should be selected such that their hybridisation properties are similar in terms of melting temperatures. The length of the polynucleotide probe will depend on the specificity requirements of the assay. For example with human genomic DNA sequences probes of at least 17 bases are preferable for reasons of specificity (Wallace et al, Proc Natl Acad Sci 80, 278, 1983). The upper limit of probe length is determined by convenience of synthesis and thus for synthetic oligonucleotides the upperlimit is conveniently about 100 bases. Sequences which have been amplified, for example by PCR, can be detected by probes shorter than 17 bases without impairing specificity. Typically probes will be in the range of 10 to 60 bases and conveniently from 20 to 30 bases.

The amount of each polynucleotide probe added is conveniently approximately equal to the amount of target. Optimisation will be required for each assay depending on the range of target concentrations to be measured. This variable will have to be considered alongside the particle concentration to ensure that the prozone phenomenon does not adversely effect the performance of the assay as discussed below. Where the target nucleic acid sequence is generated by an amplification step, for example PCR, the amplification is conveniently carried out so that the amount of amplified nucleic acid generated is approximately constant. This will minimise the range of target concentrations to be measured and thus make optimisation of the assay easier to perform.

A feature of double stranded nucleic acids is that after denaturation to separate the target strand from its complement, the complementary strand can interfere with hybridisation of probes to the target due to re-annealing to form the original duplex. In cases where the target nucleic acid has been generated by PCR this effect

can be minimised by the following techniques:

(i) By the use of relatively short PCR products, conveniently those less than 100 base pairs, the melting temperature of the original duplex is minimised.

(ii) By use of the PCR reaction to generate a single stranded product using the so-called "asymmetric PCR" as described by Gyllensten U.B. et al, Proc Natl Acad Sci 1988, 85, 7652.

(iii) By treating a double stranded PCR product with exonuclease to generate a single stranded product as described by Higuchi R.G. et al, Nuc Acids Res, 1989, 17, 5865.

For any case where re-annealing of a double stranded nucleic acid occurs it will be advantageous to hybridise probes along the full available length of the target strand to minimise the effect.

According to a further feature of the present invention we provide a method for detecting the presence or absence of a target nucleic acid sequence as hereinbefore described wherein at least one of the monovalent binding partners has been incorporated into the species by elongation of a nucleic acid sequence. In a convenient example deoxynucleoside triphosphates, one of which is labelled with a monovalent binding partner(mbp), may be used to incorporate nucleotides labelled with mbp into a PCR product. For example biotin-21-dUTP (available from Clontech, Palo Alto, California) may be incorporated in such a manner. In this example the corresponding particle may be coated with avidin, streptavidin or anti-biotin antibody. It is preferable to remove unincorporated biotin-21-dUTP before addition of the particle reagent for example by the use of microfiltration tubes such as Centricon-100 (Amicon) microfiltration tubes. The microfiltration membrane molecular weight cut-off is preferably selected such that PCR product is retained whilst unincorporated biotin-21-dUTP passes through. The use of Centricon-100 tubes to separate PCR products from at least some of the reagents used in the PCR reaction has been described by F.F. Chebab and Y.W. Kan. PNAS USA, 9178. (1989). In general it is important that the mbp used is compatible with the reaction conditions for formation of the nucleic acid polymer such that it retains its ability to bind

to the corresponding binding partner on the particle reagent and it does not significantly inhibit the f rmation of nucleic acid polymer compared with the nucleoside triphosphate without mbp attached. Another example is the production of an RNA polymer using an RNA polymerase and incorporation of mbp labelled nucleotides from nucleoside triphosphate with mbp attached such as may be applied to the transcription amplification system of SISKA Diagnostics. A further example is the production of an RNA polymer using a replicase such as Q-beta replicase and incorporation of mbp labelled nucleotides from nucleoside triphosphate with mbp attached.

According to a further feature of the present invention we provide a method for detecting the presence or absence of a target nucleic acid sequence as hereinbefore described wherein at least one of the monovalent binding partners has been incorporated into the species by extension of a polynucleotide primer comprising one copy of a monovalent binding partner. A preferred method comprises contacting the sample nucleic acid with two oligonucleotide primers, each containing one copy of a monovalent binding partner and performing PCR amplification as described above. Subsequently particles coated with the corresponding binding partner to the monovalent binding partner on the primers are added. Incorporation of the primers into a double stranded PCR product will result in agglutination of the particles which can be detected by appropriate means. The monovalent binding partner (mbp)used on the PCR primers must be compatible with the reaction conditions used in the PCR amplification. Firstly the mbp must retain its ability to bind with the binding partner onthe particles after the PCR. Secondly the mbp must not significantly inhibit the ability of the oligonucleotide primers to be acted upon by the DNA polymerase used in the PCR. Since the extension is from the 3′ end of the primer the mbp may be best situated at the 5′ end. Only a double stranded PCR product will lead to agglutination and therefore single stranded PCR products as generated by asymmetric PCR or by exonuclease treatment of double stranded PCR products is unsuitable. It is preferable to remove unincorporated PCR primers from the PCR product before addition of the particle reagent. This ensures that.

the amount of particle reagent used is minimised. This may be achieved by the use of for example microfiltration with for example Centricon-100 tubes as outlined above. Furthermore the removal of unincorporated primers may keep the amount of particle reagent used within the range of any optical instrumented measurement. Note that in a typical PCR reaction the unincorporated primers may be in a 10-fold excess over primer incorporated into PCR product.

According to a further feature of the present invention we provide a method for detecting the presence or absence of a target nucleic acid sequence as hereinbefore described wherein the species comprises only two monovalent binding partners one of which is attached to a polynucleotide probe which hybridises in an allele specific manner. In this manner the invention may be advantageously applied to the detection of mutations in a target nucleic acid sequence. A convenient example for the detection of mutations comprises the use of 2 nucleotide probes only, each with one copy of mbp, and one of the two probes being directed to the mutation locus of interest. Hybridisation conditions are then optimised such that the probe directed to the mutation locus of interest binds in an allele specific manner i.e. it binds to the locus if the mutation is present but not if it is absent, conversely if the probe is directed to the normal allele the probe binds if the normal allele is present but not if it is absent. The second probe is designed such that it binds to the target sequence regardless of the presence or absence of the mutation locus. Subsequently the particle reagent is added and agglutination occurs only if both probes have hybridised to the target sequence. For example the Phe 508 mutation responsible for some cases of the inherited disease cystic fibrosis could be examined.

According to a further feature of the present invention we provide a method for detecting the presence or absence of a target nucleic acid sequence as hereinbefore described wherein the species comprises only two monovalent binding partners separated by a restriction endonuclease site. This provides a further example of how the invention may be advantageously applied to the detection of

mutations in a target nucleic acid sequence. Restriction end nuclease sites have been used in the diagnosis of human genetic diseases (Kan Y.W. and Dozy A.N. [1978] Lancet, 2, 910-912). A convenient example comprises treatment of the species with a restriction endonuclease whose action is dependent on the presence or absence of a particular mutation in the target nucleic acid sequence before addition of the coated particles. Agglutination of the coated particles will be impaired if the restriction endonuclease has acted on the species to sever the linkage between the two monovalent binding partners.

According to a further feature of the present invention we provide a method for detecting the presence or absence of a target nucleic acid sequence as hereinbefore described wherein at least one of the monovalent binding partners has been incorporated into the species by ligation. For example a DNA ligase may be used to link an oligonucleotide comprising a mbp to the species. A particularly convenient example comprises the ligase mediated amplification system of Biotechnica International described in EP 320308 wherein of the four oligonucleotides only two comprise a single copy of a monovalent binding partner and these two must not comprise complementary nucleic acid sequences.

According to a further feature of the present invention we provide an assay kit which comprises specific binding partner coated particles and/or means for sample preparation and/or means for generation of a species comprising at least two monovalent specific binding partners as described above together with appropriate packaging and instructions for performing a method as described above according to any feature of the present invention. Preferably the assay kit comprises only one specific binding partner coated particle and means for generation of at least 2 nucleic acid species.

According to a further feature of the present invention we provide the use of only one specific binding partner coated particle for detection of the presence of absence of at least two nucleic acid sequences according to the method defined above.

Brief description of the drawings:

Figure 1 shows a diagrammatic representation of a preferred embodiment of the invention wherein target nucleic acid strands (1) have hybridised with complementary oligonucleotide probes (2) labelled with the monovalent binding partner (mbp) such as fluorescein as represented by the letter "M"(8) which has resulted in agglutination of particles (4) coated with anti-fluorescein antibodies (5).

Figure 2 shows a diagrammatic representation of a preferred embodiment of the invention wherein; (i) a target DNA strand (1) is hybridised with an oligonucleotide primer (6) and; (ii) the elongated nucleic acid sequence (7) wherein monovalent binding partners represented by the letter "M"(8) have been incorporated from mbp labelled deoxynucleoside triphosphates using a DNA polymerase to catalyse the reaction, this species can agglutinate particles coated with binding partners for the mbp.

Figure 3 shows a diagrammatic representation of a preferred embodiment of the invention wherein; (i) shows a double stranded DNA target (9) aligned with 2 oligonucleotide primers (10) labelled with monovalent binding partner represented by the letter "M"(8) and ; (ii) shows the species produced (11) after subjecting the target and primers of (i) to amplification by polymerase chain reaction, said species can agglutinate particles coated with binding partners for the mbp.

Figure 4 shows a diagrammatic representation of a preferred embodiment of the invention for the detection of mutations in a target nucleic acid sequence (1) comprising a mutation locus (12) aligned with 1 oligonucleotide probe (13)complementary to the region containing the mutation locus and another oligonucleotide probe (14)complementary to an adjacent region, both probes (13) and (14) labelled with mbp represented by the letter "M"(8). Hybridisation of both probes will result in agglutination of particles coated with binding partner for mbp, and if assay conditions are selected such that probe (13) hybridises in an allele specific manner agglutination of the particles will only result if the fully complementary sequence to probe (13) is present in the target.

Figure 5 shows a diagrammatic representation of a preferred embodiment

of the inventi n for the detection of mutation loci susceptible to digestion with a restriction endonuclease wherein a double stranded nucleic acid species (15) generated by PCR using primers labelled with mbp, said primers aligned to amplify the mutation locus of interest, and species (15) thus comprising 2 mbps represented by the letter "M"(8) separated by a restriction endonuclease site (16). Treatment of this species with an appropriate restriction endonuclease will result in the link between the 2 mbps being severed if the diagnostic site is digested and subsequent agglutination of particles coated with binding partner for the mbp will be inhibited.
Figure 6 shows a diagrammatic representation of a preferred embodiment of the invention applied to the ligase mediated amplification reaction disclosed in EP 320308A2 wherein; (i) 4 oligonucleotides (17,18,19 & 20) are aligned with a double stranded target nucleic acid sequence (21) and only oligonucleotides 18 and 19 are labelled with mbp(8) and; (ii) the species produced after ligase mediated amplification which will agglutinate particles coated with binding partner for the mbp.

The following non-limiting Examples are given by way of illustration only.

Example 1 - Preparation of latex particles coated with anti-fluorescein antibody

Anti-fluorescein antibodies (obtained from International Laboratory Services, London,UK), partially purified from sheep serum by ammonium sulphate precipitation have been passively coated onto 778nm diameter polystyrene latex particles (Rhone-Poulenc, product number K080, lot 831, 30% [w/v]) according to the method of Masson et al, Methods in Enzymology, (1981), 106. The 30% latex suspension was diluted to 10% with water. Briefly, 100ul of anti-fluorescein antibody (8.74 mg/ml), 50ul of 10% latex (w/v)and 700ul of 0.2X glycine buffered saline (GBS) buffer (1XGBS= 0.17M glycine. 0.1M sodium chloride, 0.4% sodium azide, adjusted to pH8.8 with HCl) were added to a 1.5ml Eppendorf tube which was vortexed for 30 seconds. After 30 min incubation at room

mperature the tube was centrifuged at 13500rpm for 2min, the supernatant aspirated and 1ml of 0.2X GBS was added. This wash step was repeated once with 0.2X GBS and once with 1X GBS, 1% bovine serum albumin (BSA), (GBS-BSA). The latex was stored in 1ml of GBS-BSA overnight at 4 degrees C. After sonication for 20 sec (using an Ultrasonics Inc W 375 model set to continuous, 50% duty cycle, output control set at 5) it was ready for use. The working latex suspension is a 1/28 dilution of the latex stock (@ 0.01%).

Example 2 - Preparation of fluorescein labelled oligonucleotide probes

5'(aminohexylphosphoryl) oligonucleotides were labelled with fluorescein isothiocyanate (FITC) according to a method (with modifications) described in "DNA modification reagents for use in automated DNA synthesis, A users manual" (1989), pg12, published by Clontech. 200µl of the crude 1µmole synthesis scale oligonucleotide (@ 20 OD units - synthesised on an Applied Biosystems automated DNA synthesiser using protocols recommended by the manufacturer) was made up to 900µl with deionised water. 100µl of 1M sodium carbonate, sodium bicarbonate buffer pH7 was added and the mixture vortexed. 250µl of a freshly prepared 100mg/ml FITC in dimethylformamide was added to the oligonucleotide solution, mixed well and incubated overnight in the dark at room temperature. (Note if the solution had precipitated overnight, 250µl of 0.1M sodium bicarbonate, sodium carbonate buffer pH9 was added and the mixture incubated at 42 degrees C until the precipitate redissolved. The mixture was the desalted down a Nap-25 column using pH7 carbonate buffer.) The fluorescein labelled oligonucleotide was then purified by reverse phase high performance liquid chromatography(HPLC) on a RP-C18 analytical column (4.6mm X 250mm) from Waters Chromatography. The gradient was from 0.1M triethylammonium acetate to 90% acetonitrile in 0.1M triethylammonium acetate. The purified oligonucleotide was desalted by passing it down a Nap 25 column (Pharmacia) and eluting in deionised water.

Example 3 - Hybridisation conditions

Hybridisations have been carried out using oligonucleotide targets and equimolar concentrations of oligonucleotide probes as described below:

In 100µl of GBS-BSA buffer add 100pmoles of target nucleic acid sequence and 100pmoles of each fluorescein labelled oligonucleotide probe. Incubate for 30min at room temperature to allow hybridisation to occur rapidly at high DNA concentration. The hybridised solution was then diluted in GBS-BSA 4-fold to give a target and probe concentration of 25 pmoles/100µl and then added to 1ml of working latex suspension to give a final target and probe concentration of 2.27pmol/100µl and the optical density was read at 405nm in a Phillips PU 8700 uv/vis spectrophotometer at time 0 and again 2-3 hours later. The 4-fold dilution of the hybridised solution was serially diluted in 2-fold steps and treated similarly to give final target and probe concentrations of 1.14, 0.57, 0.28 and 0.14pmol/100µl. With this assay design agglutination of the particles produces a decrease in optical density.

Example 4 - Experiment to detect agglutination of latex particles in the presence of a 60mer oligonucleotide target and 4 15mer fluoresceinlyated oligonucleotide probes.

The assay was performed as described in example 3. The 60mer target (SEQ ID NO:1) was incubated with 4 15mer probes (SEQ ID NOS:5, 2, 4 & 3 labelled with fluorescein according to Example 2). Tubes 2-6 contain target and probes, tube 1 contains neither target nor probe as a control, tubes 7-11 contain no target as controls and tubes 11-16 contain no probes as controls.

Tube	Target(pmol)	Probe(pmol)	OD 0min	OD 147min	Change in OD
1	0	0	2.062	2.055	0.007
2	2.27	2.27	2.067	1.998	0.069
3	1.14	1.14	2.058	1.994	0.064
4	0.57	0.57	2.068	2.019	0.049
5	0.28	0.28	2.063	2.035	0.028
6	0.14	0.14	2.070	2.057	0.013
7	0	2.27	2.063	2.048	0.015
8	0	1.14	2.068	2.053	0.012
9	0	0.57	2.067	2.055	0.009
10	0	0.28	2.074	2.065	0.009
11	0	0.14	2.069	2.066	0.003
12	2.27	0	2.066	2.065	0.001
13	1.14	0	2.073	2.069	0.004
14	0.57	0	2.072	2.069	0.003
15	0.28	0	2.072	2.069	0.003
16	0.14	0	2.074	2.071	0.003

Note that agglutination occurs in the presence of both target nucleic acid sequence and probes but to a significantly lesser extent in the absence of one or the other or both.

Example 5 - Experiment to test for agglutination in the presence of a non-complementary target nucleic acid sequence

The experiment was set up as indicated below to compare agglutination in the presence of a target oligonucleotide (SEQ ID NO:1) complementary to the fluoresceinlyated probes (SEQ ID NOS:2,3,4,5) with a non-complementary target oligonucleotide (SEQ ID NO: 6).

Tube	oligo 1 (pmol)	oligo 6 (pmol)	oligo probes (pmol)	OD 0min	OD 145min	Change OD
1	2.27	0	2.27	2.133	2.077	0.056
2	1.14	0	1.14	2.132	2.066	0.066
3	0.57	0	0.57	2.136	2.073	0.063
4	0	2.27	2.27	2.134	2.100	0.034
5	0	1.14	1.14	2.137	2.121	0.016
6	0	0.57	0.57	2.143	2.129	0.014
7	0	0	2.27	2.139	2.135	0.004
8	0	0	1.14	2.139	2.131	0.008
9	0	0	0.57	2.141	2.133	0.008

Note that agglutination occurs to a lesser extent in the presence of non-complementary target compared with complementary target.

Example 6 - Experiment to compare agglutination in the presence of 2, 3 or 4 fluoresceinylated probes.

The experiment was performed using 2 (SEQ ID NOS:2, 3), 3 (SEQ ID NOS:2, 5, 3) or 4 (SEQ ID NOS:2, 5, 4, 3) fluoresceinylated oligonucleotide probes.

Tube	Number of Probes	Target (pmol)	Probe (pmol)	OD 0min	OD 111min	Change in OD
1	4	1.14	1.14	2.065	1.934	0.131
2	4	0	1.14	2.070	2.057	0.013
3	3	1.14	1.14	2.070	1.978	0.092
4	3	0	1.14	2.071	2.056	0.015
5	2	1.14	1.14	2.073	2.019	0.054
6	2	0	1.14	2.075	2.058	0.017
7	0	0	0	2.071	2.072	0.001

Note that the signal to noise ratio improves as the number of probes is increased from 2 to 4. Nonetheless even with 2 probes significant agglutination has occured.

Example 7 - Detection of a PCR product using 3 fluoresceinylated oligonucle tide probes

The target in this example was a 400 base PCR product from the amplification of a region of the major outer membrane protein (MOMP) sequence in Chlamydia trachomatis (serovar L2).
PCR conditions: 1µl of proteinase K treated L2 cell lysate was mixed with 100µM deoxynucleoside triphosphates, 100pmoles of primer 8(see appendix for primer sequences), 1pmole of primer 7, 2.5 units of Taq polymerase in 100µl 1X Taq polymerase buffer. The reaction was for 45 cycles with the denaturing step for 45 s at 94°C, annealing for 45 s at 55°C and extension for 90 s at 72°C.
Hybridisations of PCR product with 3 fluoresceinylated oligonucleotides (SEQ ID NOS: 5, 3 and 4) in a total volume of 30µl were for 2h at room temperature before addition to the latex suspension as indicated below.
Other details of the hybridisation were as for Example 3.
The experiment was set up as indicated below:

Target (µl of PCR reaction hybridised with probes)	Probes (pmol added to 1.1ml of working latex suspension)	OD(0min)	OD(overnight)	Change in OD
5	0.5	1.828	1.603	0.226
0	0.5	1.823	1.735	0.088
2.5	0.25	1.822	1.651	0.171
0	0.25	1.825	1.752	0.073

Note that there is a significant increase in the OD change in the presence of target.

SEQUENCE LISTING

INFORMATION FOR SEQ ID NO: 1

(i)SEQUENCE CHARACTERISTICS:

LENGTH:60 bases
TYPE: nucleotide
STRANDEDNESS: single
TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION SEQ ID NO:1

TTGCCGCTTT GAGTTCTGCT TCCTCCTTGC AAGCTCTGCC TGTGGGGAAT CCTGCTGAAC 60

INFORMATION FOR SEQ ID NO: 2

(i)SEQUENCE CHARACTERISTICS:

LENGTH:15 bases
TYPE: Nucleotide (fluorescein labelled at 5')
STRANDEDNESS: Single
TOPOLOGY: Linear

(ii) SEQUENCE DESCRIPTION SEQ ID NO:2

AACTCAAAGC GGCAA 15

INFORMATION FOR SEQ ID NO: 3

(i)SEQUENCE CHARACTERISTICS:

LENGTH:15 bases
TYPE: Nucleotide (fluorescein labelled at 5')
STRANDEDNESS: Single
TOPOLOGY: Linear

(ii) SEQUENCE DESCRIPTION SEQ ID NO:3

GCAAGGAGGA AGCAG 15

INFORMATION FOR SEQ ID NO: 4

(i)SEQUENCE CHARACTERISTICS:

LENGTH:15
TYPE: Nucleotide (fluorescein labelled at 5')
STRANDEDNESS: Single
TOPOLOGY: Linear

(ii) SEQUENCE DESCRIPTION SEQ ID NO:4

CCACAGGCAG AGCTT 15

INFORMATION FOR SEQ ID NO: 5

(i)SEQUENCE CHARACTERISTICS:

LENGTH:15 bases
TYPE: Nucleotide (fluorescein labelled at 5')
STRANDEDNESS: Single
TOPOLOGY: Linear

(ii) SEQUENCE DESCRIPTION SEQ ID NO:5

GTTCAGCAGG ATTCC 15

INFORMATION FOR SEQ ID NO: 6

(i)SEQUENCE CHARACTERISTICS:

LENGTH:65 bases
TYPE: Nucleotide
STRANDEDNESS: Single
TOPOLOGY: Linear

(ii) SEQUENCE DESCRIPTION SEQ ID NO:6

CCCTCTCTCT CCCTTTCCTC CTTCCTTTCC TTCCCCTCTT CTAGTTGGCA TGCTTTGATG 60
ACGCT 65

INFORMATION FOR SEQ ID NO:7

(i)SEQUENCE CHARACTERISTICS:

LENGTH:25 bases
TYPE: Nucleotide
STRANDEDNESS: Single
TOPOLOGY: Linear

(ii) SEQUENCE DESCRIPTION SEQ ID NO:7

TGAAATCGGT ATTAGTGTTT GCCGC

25

INFORMATION FOR SEQ ID NO: 8

(i)SEQUENCE CHARACTERISTICS:

LENGTH:25 bases
TYPE: Nucleotide
STRANDEDNESS: Single
TOPOLOGY: Linear

(ii) SEQUENCE DESCRIPTION SEQ ID NO:8

TGGTGGCTCC TAATGTACAG AATAC

25

CLAIMS

1. A method for detecting the presence or absence of a target nucleic acid sequence in a sample by particle agglutination which method comprises contacting specific binding partner coated particles which do not directly hybridise to the target nucleic acid sequence with a species comprising nucleic acid which species is formed as a function of the presence or absence of the target nucleic acid and which also comprises at least two monovalent binding partners which may be the same or different and detecting the presence or absence of particle agglutination mediated by said species.

2. A method according to claim 1 wherein at least one of the monovalent binding partners has been incorporated into the species by hybridisation of a polynucleotide probe comprising one copy of a monovalent binding partner.

3. A method according to claim 1 wherein at least one of the monovalent binding partners has been incorporated into the species by elongation of a nucleic acid sequence.

4. A method according to claim 1 wherein at least one of the monovalent binding partners has been incorporated into the species by extension of a polynucleotide primer comprising one copy of a monovalent binding partner.

5. A method according to claim 1 wherein the species comprises only two monovalent binding partners one of which is attached to a polynucleotide probe which hybridises in an allele specific manner.

6. A method according to claim 1 wherein the species comprises only two monovalent binding partners separated by a restriction endonuclease site.

7. A method according to claim 1 wherein at least one of the monovalent binding partners has been incorporated into the species by

ligation.

8. An assay kit which comprises specific binding partner coated particles and/or means for sample preparation and/or means for generation of a species comprising at least two monovalent specific binding partners as described above together with appropriate packaging and instructions for performing a method defined in claim 1.

9. The use of only one specific binding partner coated particle for detecting the presence of absence of at least two target nucleic acid sequences according to the method defined in claim 1.

Fig. 1.

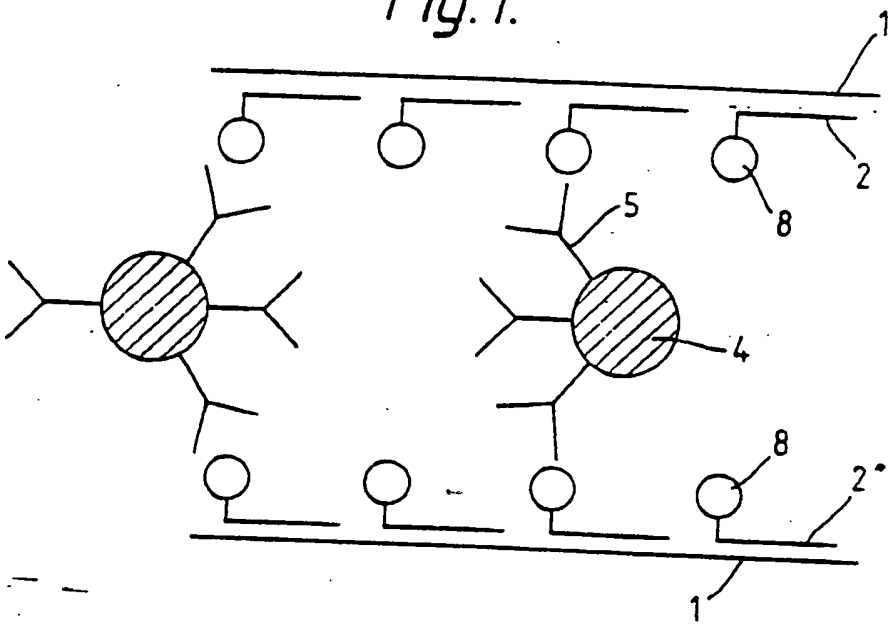


Fig. 2.

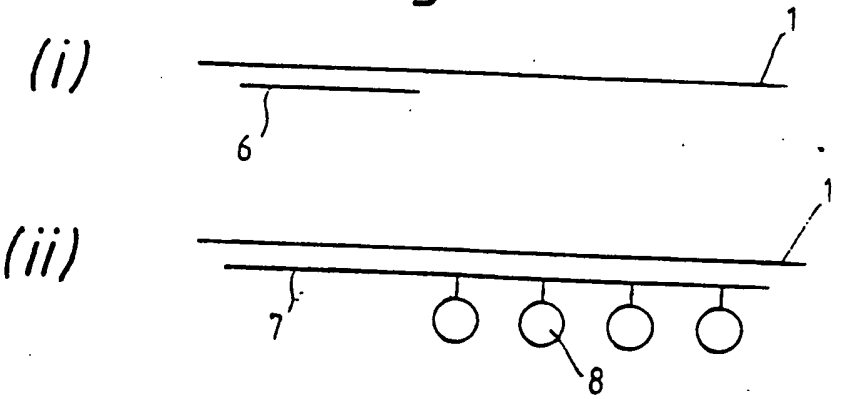


Fig. 3.

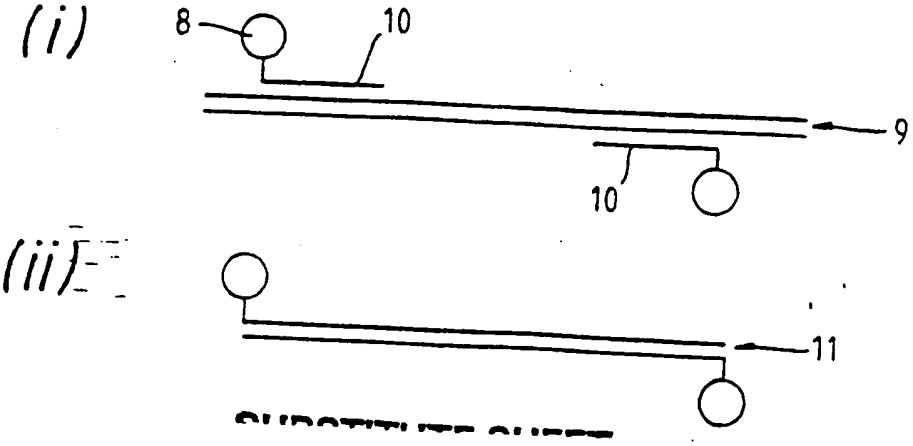


Fig. 4.

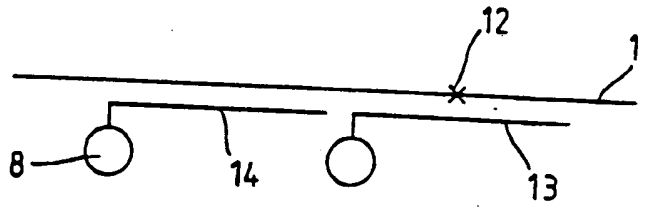


Fig. 5.

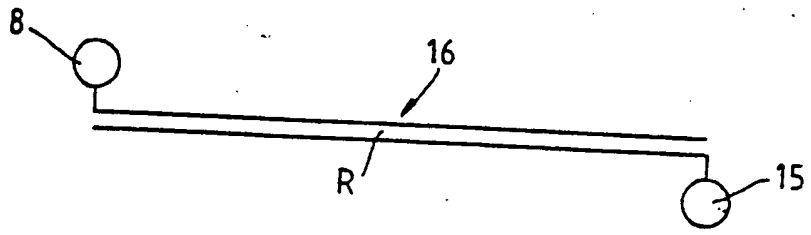
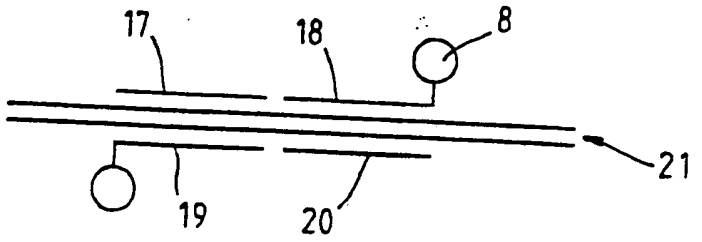


Fig. 6.

(i)



(ii)